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To test if Neu differentiation factor (NDF), an activator of the c-*neu* gene, is proliferative or differentiative in the mammary gland, we have generated a transgenic mouse line overexpressing NDF in the mammary epithelium. These mice develop mammary adenocarcinomas in a stochastic fashion, develop Harderian gland hyperplasia and have subtle developmental abnormalities of the mammary gland. Together, these data point to a proliferative effect of NDF in the mammary gland and possibly an inhibitory effect on apoptosis.

The role of the c-*neu* proto-oncogene in normal development is unclear, though its expression is seen in a variety of fetal tissues. We have used homologous recombination and gene targeting strategies to disrupt the c-*neu* gene to understand its potential role in development. Analysis of several generations of these animals has revealed that the c-*neu* gene is vital to development. No animals carrying two copies of the mutated gene develop to term. Homozygous mutants do not live past day 10 of gestation. Studies are ongoing to determine the mechanism by which the absence of Neu can lead to premature death.

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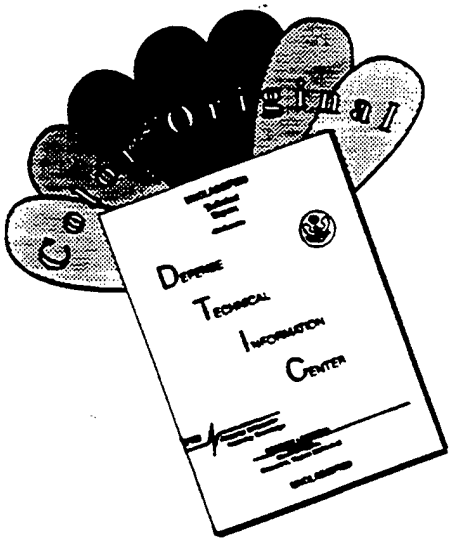

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Introduction

In recent years, a family of stimulatory ligands for the EGF/*erb*B family of receptor tyrosine kinases has been described. This family includes Neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor inducing activity (ARIA), and the glial growth factors (GGFs)(Falls et al., 1993; Holmes et al., 1992; Marchionni et al., 1993; Wen et al., 1992). This family of ligands, known collectively as the neuregulins, is comprised of alternatively spliced isoforms of a common gene. Interaction of the products of this gene with heterodimers and/or homodimers of the *erb*B receptors leads to their auto and trans-phosphorylation and subsequent interaction with SH-2 proteins including phospholipase C-γ (PLC-γ), the p85 subunit of posphotidylinositol 3'-kinase (PI-3 K), and the GTPase activating protein of *ras* (*ras*-GAP)(Peles et al., 1991).

The EGF receptor (EGFR) has been associated with cancers of the breast, bladder, lung and stomach. Another member of the EGFR family, HER2/*erb*B2/c-*neu*, has been implicated in a large proportion of human malignancies, including breast and colon carcinomas, and its amplification and/or overexpression has been associated with a poor prognosis(Slamon et al., 1987). Our laboratory has demonstrated that the overexpression of an activated form of this receptor, when targeted to the murine mammary gland using the mouse mammary tumor virus promoter (MMTV), is transforming(Muller et al., 1988). Overexpression of the cellular form of this receptor in the mammary gland is also transforming, though in a more stochastic fashion(Guy et al., 1992).

Binding of NDF to the cell surface through one or more of the *erb*B receptors is thought to stabilize receptor dimers, similarly to the effect produced by the point mutation found in the transmembrane domain of the activated *neu* oncogene, a mutation not found in human tumors(Bargmann and Weinberg, 1988). Although the role of NDF in breast cancer is still unclear, its biological effects on mammary epithelial cells *in vitro* are quite profound. Some mammary epithelial cell lines can be stimulated to proliferate more rapidly, while others enter G_0 arrest in response to NDF, and begin to exhibit markers of mammary cell differentiation such as casein and lipid production(Holmes et al., 1992; Peles et al., 1992). Elevated expression of NDF in a number of human mammary tumor cell lines has

been shown to correlate with the expression of vimentin, a marker of metastatic disease; and it has also been demonstrated that the human breast epithelial cell line, MCF-7, which is unable to form tumors in nude mice, will form tumors when stably transfected with NDF, the growth of which can be inhibited by anti-NDF antibodies (R.Lupu, personal communication).

We have created a transgenic mouse model to test if the overexpression of NDF in the mouse mammary gland can lead to the transformation of the mammary epithelium, or lead to other, differentiative, effects on mammary gland development. Much of that work has been published recently(Krane and Leder, 1996). We have also targeted the disruption of the c-*neu* proto-oncogene to examine its potential role in development of the mouse as a whole as well as its mammary gland development.

Body

NDFβ2 transgene construct and tissue-specific expression

In order to study the biological effects of high levels of NDF in the breast, we have created transgenic mice overexpressing NDF mRNA in the mammary epithelium. A recombinant plasmid containing the MMTV LTR fused to the murine β2c NDF isoform cDNA was microinjected into the male pronucleus of a one-cell mouse embryo. Ligated to the MMTV promoter/enhancer is a 1.2 kb cDNA containing the entire coding sequence of murine β2c NDF isolated from a v-Ha-*ras* -induced murine mammary gland tumor using the polymerase chain reaction (PCR). This *ras*-induced tumor(Sinn et al., 1987) was chosen as a tissue source of NDF as the original NDF clone descibed was isolated from a *ras*-transformed RAT-1 fibroblast cell line(Wen et al., 1992). To ensure proper expression of this cDNA, simian virus 40 (SV40) splicing and polyadenylation signals were added to the 3' end of the transgene construct.

Three transgenic founder animals carrying the fusion gene were generated (TG.IJ, TG.IK, and TG.IM), and each animal passed the transgene to its progeny in a Mendelian fashion. The best characterized line, TG.IJ, will be described here.

The SV40 fragment, when used as a probe, gives similar results to the NRG probe in Southern (Fig. 1) and Northern analyses, though it is preferred to the NRG probe because it does not recognize the endogenous gene or the multiple endogenous NRG mRNA transcripts (data not shown). The tissue-specific expression of the transgene was determined by Northern blot analysis of total RNA isolated from a variety of organs (Fig. 2). The probe used in this analysis was an SV40-fragment specific to the transgene construct. Interestingly, highest levels of transgene expression are seen in the salivary gland of virgin animals, a gland rarely transformed by overexpression of other oncogenes using the MMTV LTR.

NDFβ2 transgene expression leads to Harderian gland hyperplasia

Approximately 50% of all TG.IJ animals, male and female, exhibit a unilateral or bilateral exophthalmous resulting from a progressive enlargement of the Harderian gland, a tubuloalveolar gland located within the orbit of many terrestrial species, though absent in primates. This phenotype is observed as early as the time of weaning (3 weeks of age), and is coincident with transgene

expression, as animals exhibiting unilateral exophthalmous show no detectable transgene expression in the unaffected contralateral gland, but demonstrate considerable levels of transgene messenger RNA in the affected gland, higher even than those seen in the mammary gland (Fig. 3). After histologic evaluation, these enlarged Harderian glands are characterized as hyperplastic adenomas. While growing *in situ*, they do not invade the bone of the surrounding orbit, and they fail to grow when transplanted into a syngeneic host indicating they are not transformed.

Persistence of terminal end bud structures

We studied possible effects of transgene expression in the developing mammary gland by examining ductal morphogenesis in these animals. Although pregnancy and lactation stimulate the highest levels of transgene expression from the MMTV LTR in the mammary gland, we have demonstrated that the MMTV-NDF transgene is clearly expressed in virgin glands. To determine if there are any developmental abnormalities associated with transgene expression in these virgin glands, we prepared mammary gland whole mounts stained with carmine red alum to examine the growth of the mammary gland ductal tree (Fig. 4)(Sympson et al., 1994). In the normal developing mouse mammary gland, as the animal passes through sexual maturity, ductal epithelial structures gradually fill the mammary fat pat in response to mesenchymal signals(Sakakura et al., 1976). The terminal end bud in the developing gland functions as a growth point, driving ductal morphogenesis by providing differentiated ductal and myoepithelial cells for the formation and elongation of secondary ducts. In a wildtype virgin female, these multi-layered terminal end bud structures have virtually disappeared by ten weeks of age, as the multiple layers of end bud cells undergo an apoptotic regression(Strange et al., 1992). We sacrificed a number of virgin transgenic female mice at various developmental stages and found that, although the mammary glands of younger animals appeared normal, glands from older animals (>10 weeks) had increased numbers of TEBs as compared with age-matched control animals.

Stochastic appearance of breast tumors NDF transgenic mice

To examine the potential ability of the MMTV-NDF transgene product to elicit a tumorigenic phenotype in the mice, female TG.IJ transgenic mice were set up to breed continuously, upon reaching sexual maturity, in order to maximize expression of the transgene from the MMTV LTR. After the first

round of pregnancy, animals were monitored weekly for the appearance of tumors. By 14 months of age, each animal in the study had developed at least one mammary gland tumor, with the median age of tumor onset being 357 days after birth (Fig. 5). Examined histologically, all tumors were similar in nature and distinct from other MMTV-oncogene-derived mammary gland tumors described previously in our laboratory(Cardiff et al., 1991). Each tumor was characterized as an adenocarcinoma with a squamous cell component, highlighted by abundant keratinaceous debris. Northern blot analysis of total RNA from these tumors confirms high levels of transgene expression, though the mRNA source is naturally more homogeneous than the "unaffected" contralateral mammary glands typically used for comparison (Fig. 6).

These tumors continue to grow well when transplanted into the mammary fat pad of syngeneic hosts and can be adapted to cell culture conditions. One such cell line, IJ9921, was examined for *erb*B receptor expression and tyrosine phosphorylation status by immunoprecipitation and western blotting. Although each of the four known *erb*B receptors is expressed in this cell line, apparently only *erb*B3 is phosphorylated (Fig. 7). *erb*B3 is thought to have little intrinsic kinase activity in its human form, though it is active in rodent form due to amino acid changes in the tyrosine kinase domain. It remains to be determined if the other receptors may be involved in its trans-phorphorylation in these tumors.

We have recently observed through western blotting of IJ9921 cell extracts, that there are two "a"-immunoreactive forms of neuregulin synthesized in these cells, one of which is found only in the cytoplasm the other found only in the nucleus (Fig. 8). Taking into account the antibody epitope (extreme C-terminus) and the size of the proteins (~40 kDa), these reactive species are consistent with the processed intracellular domains of the "a" neuregulins. A cDNA expression library from this cell line has been made and we are currently screening it for clones which may ultimately be localized to the nucleus or cytoplasm.

The c-neu proto-oncogene is vital to murine development

In addition to the study of the biological effects of NDF in the mammary gland through stimulation of the *erb*B receptor complex, we were interested in the role of one of these receptors, *erb*B2, in development. We created a targeting construct in which the exon encoding the transmembrane domain of the receptor was removed and replaced with a neomycin resistance cassette

(including in-frame stop codons), making what we predicted would be a null mutation of the *neu* gene. An embryonic stem cell clone carrying the disrupted gene was injected into blastocysts and several chimeric animals were isolated. These animals carried the disrupted allele and were able to pass it through the germ line. Homozygous null mutants fail to develop past day 9.5-10.5 p.c. These animals fail to develop trigeminal ganglia and are thought to lack cardiac trabeculae (Lee et al., 1995) which is the proposed cause of embryonic lethality. We have observed, however, that cardiac trabeculation can be seen in homozygous nulls (Fig. 9) and, in fact, the apparent lack of trabeculation is often seen in wild-type littermates indicating the lack of trabeculae may be simply an artifact of the dissection and fixation of the cardiac tissue.

We are also currently crossing the targeted animals into a homogeneous genetic background (FVB/n), as it has been observed in the case of the EGF receptor knockout that genetic background profoundly affects the timing of its embryonic lethality(Sibilia and Wagner, 1995; Threadgill et al., 1995). We are hopeful of delaying the onset of embryonic lethality in order to better understand the underlying cause of the embryonic death.

Conclusions

The *erb*B2 gene has been shown to be transforming *in vitro* by overexpression, truncation, or point mutation. Our laboratory has demonstrated that the activating point mutation is transforming *in vivo* using a transgenic mouse model. The same mouse model has been used to show that the cellular form of *erb*B2 (c-*neu*) is transforming as well, though with a slower onset of tumor formation. We initiated this study to determine if the same transgenic model could be used to assay the proliferative potential of Neu differentiation factor, a molecule known to stimulate the *erb*B2 receptor through a heterodimeric receptor complex formed with other members of the EGF receptor family. In vitro experiments with NDF on different target mammary epithelial cell lines have yielded somewhat ambiguous results, though different responses to NDF may be a function of the complexity of the receptor types expressed on the surface of these cells. We hypothesized that if a normal receptor such as Neu, when overexpressed in the mammary gland, can provide an initiating event for tumorigenesis, then perhaps the overexpression of a gene product known to activate this receptor would have a similar function. The results of our experiments support this hypothesis in three ways. First, each animal in the study devloped a mammary tumor; in some cases two or three independent tumors were found upon necropsy. Second, there is an incompletely penetrant phenotype in which there is a massive hyperplasia of the Harderian gland in approximately 50% of these mice. These hyperplastic and hypertrophic adenomas are benign in that they are non-invasive and they fail to grow when transplanted into syngeneic host animals. Clearly, elevated levels of NDF expression is not sufficient, as high levels of transgene expression are seen in the salivary gland, yet no histolopathological consequences of this expression in that organ has ever been observed. The benign attributes of these growths imply that there are factors missing from the Harderian gland necessary for this hyperplasia to progress to a malignant neoplasm. Among these putative factors could be the right combination *erb*B receptor subtypes (*erbB*1-4). Interestingly, this Harderian gland phenotype has been seen in another transgenic mouse line in our laboratory, the MMTV-*ras* line, *ras* being a gene found to lie on the Neu signalling pathway. In that line, the glands are also hyperplastic and not neoplastic. Taken together with the NDF

transgenic animals, these data suggest that there may be factors downstream of Neu and *ras* which influence the susceptibility of a cell type to oncogenic transformation. A second, parallel, pathway may also provide the "second hit" necessary for oncogenesis and this pathway may be active in the mammary epithelium but inactive in the Harderian gland. It is also possible that there are factors inhibitory to the *neu-ras* signalling pathway which are present in the Harderian gland but absent in the mammary gland.

Lastly, a more subtle phenotype observed in the MMTV-NDF animals is that of the persistence of terminal end bud structures in the mammary gland of virgin females. The terminal end buds normally provide a source of differentiated myoepithelial cells at the growth points of the mammary ductal tree as it responds to mesenchymal signals to fill the mammary fat pad in the developing gland. These signals provide the TEBs with spatial and temporal growth cues, to avoid overgrowth of ductal structures as well as providing information to halt growth when the outer limits of the fat pad have been reached. Concomitant with the cessation of ductal growth, comes regression of the TEBs in a muture mouse. In our transgenic animals, however, the communication between the developing ductal structures and stromal signalling cells appears to be perturbed in that upon reaching the limits of the fat pad, TEBs do not consistently undergo the apoptotic regression seen in wild type animals. This is consistent with the observation of others that glial growth factor can inhibit apoptosis in Schwann cell cultures. Moreover, in mature transgenic females, TEBs are evident in regions of the mammary gland in which they are not normally seen, in the proximal regions of the gland (close to the lymph node in the #4 gland). There also appears to be a disruption in the signalling involving the direction of growth of the ducts, in that some ducts are found to have reversed direction and, in some cases, they have overlapped other ducts. These results suggest NDF expression overcomes inhibitory signals provided by the mammary stroma, not simply those signals which inhibit the growth of the ducts, but those which signal the direction of growth and those which influence the active regression of the terminal end buds. These potential inhibitory signals are overcome by a normal pregnancy, lactation and regression, as persistent TEBs are not observed in the mammary glands of such animals.

The second part of our work, discussed here, describes the embryonic lethality of generating a null mutation in the c-*neu* gene. It is obvious that this gene is

necessary for the proper development of the mouse, and we are currently searching for the mechanism by which the absence of Neu is lethal to the developing animal. Although others have recently published the targeted disruption of the c-*neu* gene, and suggest a lack of cardiac trabeculation leads to embryonic lethality, this is not consistent with our observations. It is possible however that these differences may be due to differences in gene targeting constructs, though we have seen no evidence of the production of a c-*neu* mRNA or a truncated Neu protein (data not shown).

The third part of our proposal concerns our desire to express NDF recombinantly to test its potential growth arresting effects on mammary carcinoma cell lines. We have been unable to generate sufficiently bioactive recombinant product, but we feel that we may be able to use the tumor cell line derived from our MMTV-NDF transgenic mouse line to purify such a product. We are also currently using the established tumor cell line to examine an isoform of neuregulin not known to be associated with any specific biological function, the cytoplasmic domain of the "a" isoforms.

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Figure Legends

Figure 1. MMTV-neuregulin Transgene Construct.
Above, a schematic representation of the MMTV-NRG transgene. The β2c isoform of neuregulin (NRG β2c), amplified from a *ras*-transformed tumor, was fused to the mouse mammry tumor virus promoter (MMTV) followed by the simian virus 40 splicing and polyadenylation signals (polyA). Below is a Southern blot of mouse tail DNA restricted with BamHI and probed with a full length ^{32}P-labeled NRG cDNA. In addition to the endogenous NRG gene (high MW forms in both lanes), is a smaller (~2.1 kb) fragment in the second lane corresponding to the transgene, present as a single copy.

Figure 2. Northern Blot Analysis of Transgene Expression.
Ten μg total RNA from the tissues indicated were electrophoresed through a 1.2% agarose/formaldehyde gel. Hybridization was to a SV40 polyA-specific ^{32}P-labeled cDNA probe. Transgene-specific mRNAs are indicated by the arrow. The ethidium bromide stained gel is shown to demonstrate that the gel was evenly loaded.

Figure 3. Northern Blot Analysis of Harderian Gland Hyperplasias.
Ten μg total RNA from normal and hyperplastic Harderian gland from a male mouse (first two lanes) and mammary gland and hyperplastic Harderian gland from a female mouse (last two lanes) were analyzed by Northern blotting and probed as previously described.

Figure 4. Mammary Gland Whole Mounts.
Carmine red stained mammary gland whole mount preparations of 5 month old virgin wild-type (WT) and transgenic (TG) female mice, and regressed glands from one year old animals having undergone multiple rounds of pregnancy, lactation and regression (1 yr multiparous).

Figure 5. NRG-induced Tumor Onset.
Age of each animal, in days, at the appearance of a mammary gland tumor (x-axis), versus the number of tumor-free animals in the study (y-axis). The median age of tumor onset was 357 days.

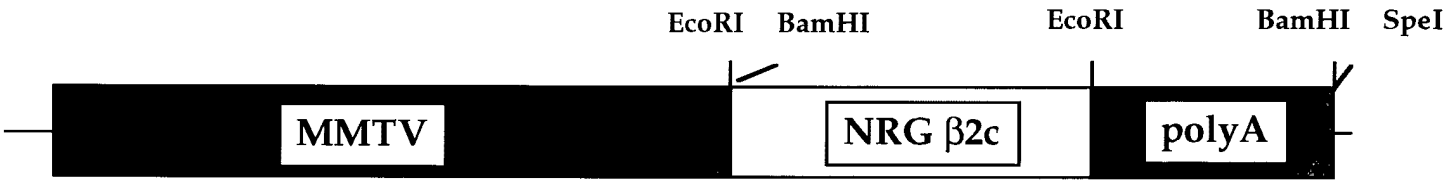
Figure 6. Northern Blot Analysis of Mammary Gland Tumors.
Ten μg total RNA from normal mammary gland (MG), mammary gland tumors (TU) and affected Harderian gland (HG) were examined as described in previous figure legends.

Figure 7. Immunoblot Analysis of ErbB Receptor Proteins in a NRG-induced Mammary Gland Tumor Cell Line.
Total cell lysates or cell lysates immunoprecipitated with anti-Neu, ErbB3, or ErbB4 antibodies as indicated were run through a 5% SDS-PAGE gel. The gel was blotted to a nylon membrane and hybridized with the anti-phosphotyrosine antibody 4G10 (anti-P tyrosine, UBI), stripped and re-probed with the anti-Neu antibody (anti-Neu, Ab3, Oncogene Science) and stripped a second time and reprobed with an anti-erbB4 antibody (anti-ErbB4, Santa Cruz). The NF tumor cell line was derived from a MMTV-neu oncogene-induced mammary gland tumor (Muller et al., 1988) and the NRG-induced tumor cell line was established from the IJ9921 mammary gland tumor (see Figure 6).

Figure 8. Western Blot Analysis of Subcellular Fractions With "a" Isoform-specific Antibody.
Subcellular fractions of the 9921 cell line were analyzed by Western blotting. Above an immunoblot of a 10% SDS-PAGE gel hybridized with the polyclonal antibody HRG-a (Santa Cruz). Below, the same fraction were analyzed with the anti-Neu antibody (Ab3) to demonstrate the nuclear contained no membrane protein. The arrows show the two reactive bands found in either the nucleus or cytoplasm.

Figure 9. Immunohistochemistry of Day 10.5 Neu Homozygous Mutant Embryo.
Fluorescent staining of the heart of a homzygous null (-/-) mutant c-*neu* knockout mouse with an anti-lectin antibody which labels endothelial cells. Arrow indicates trabeculae in the common ventricle (V). No such structures are seen in the common atrium (A).

NEUREGULIN TRANSGENE CONSTRUCT



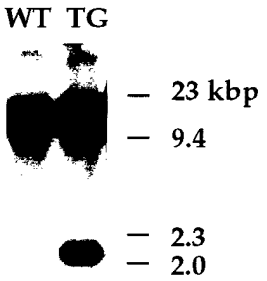
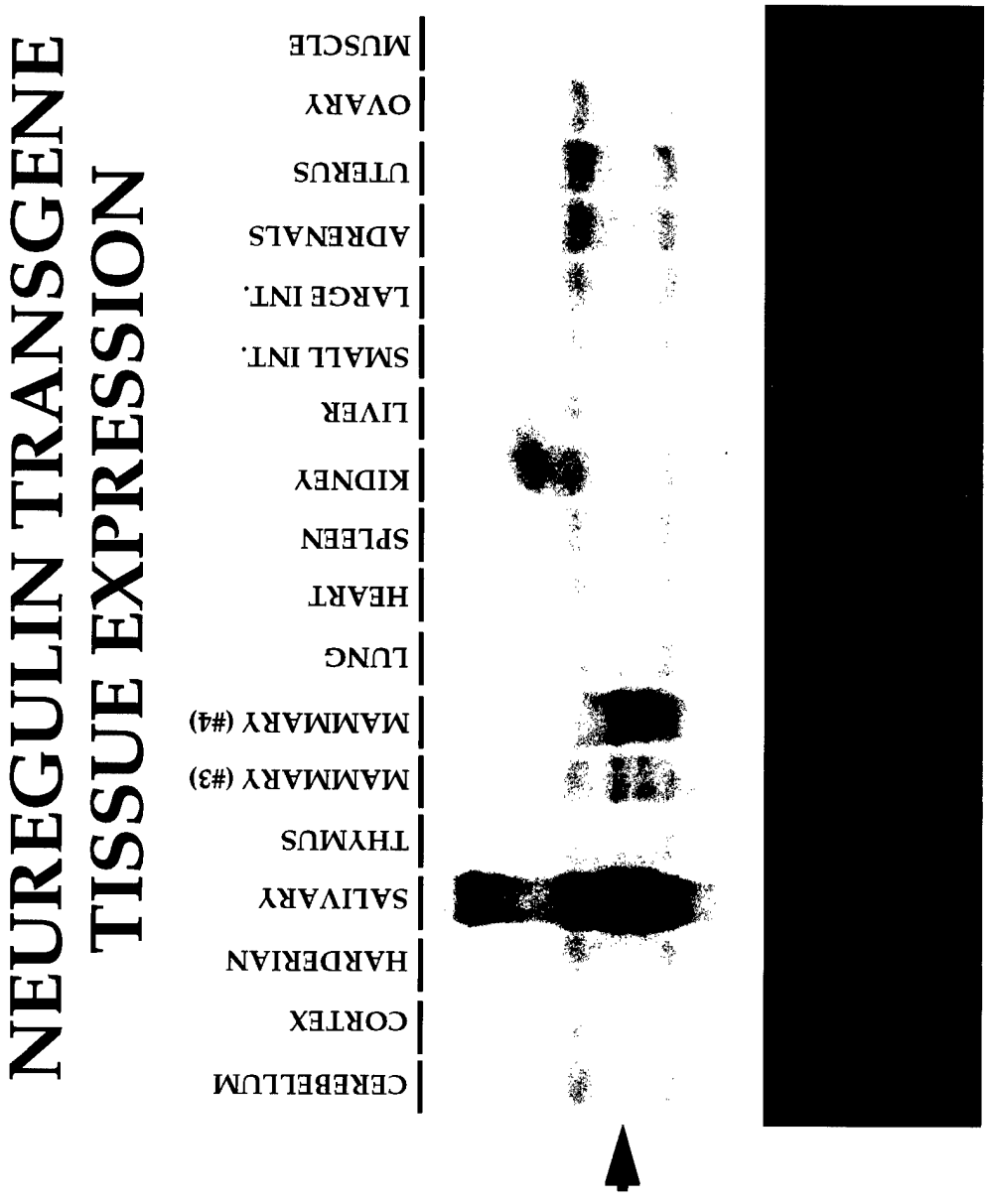


Figure 1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MUSCLE

Figure 2

NEUREGULIN TRANSGENE OVEREXPRESSION IN AFFECTED HARDERIAN GLANDS

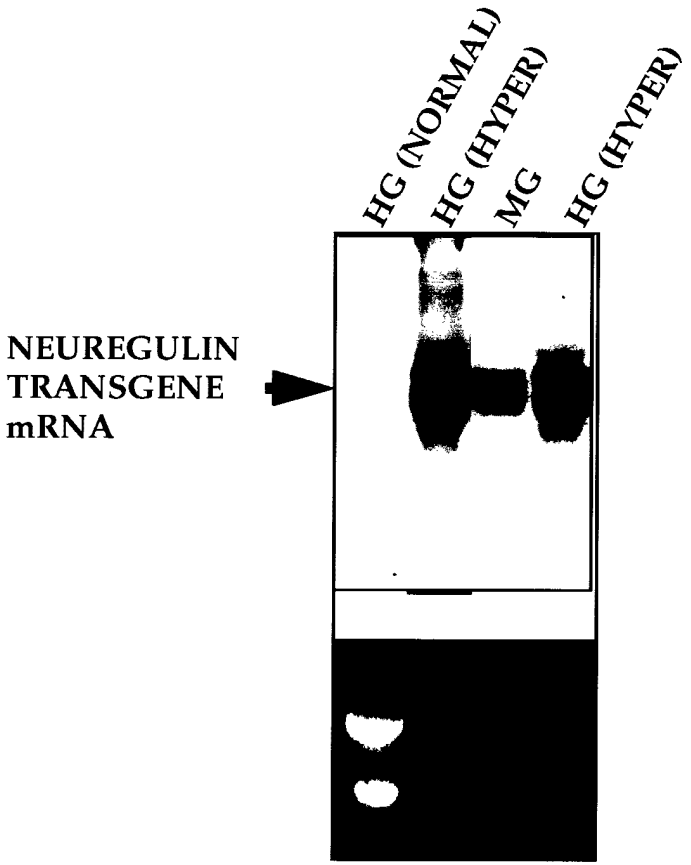

Figure 3

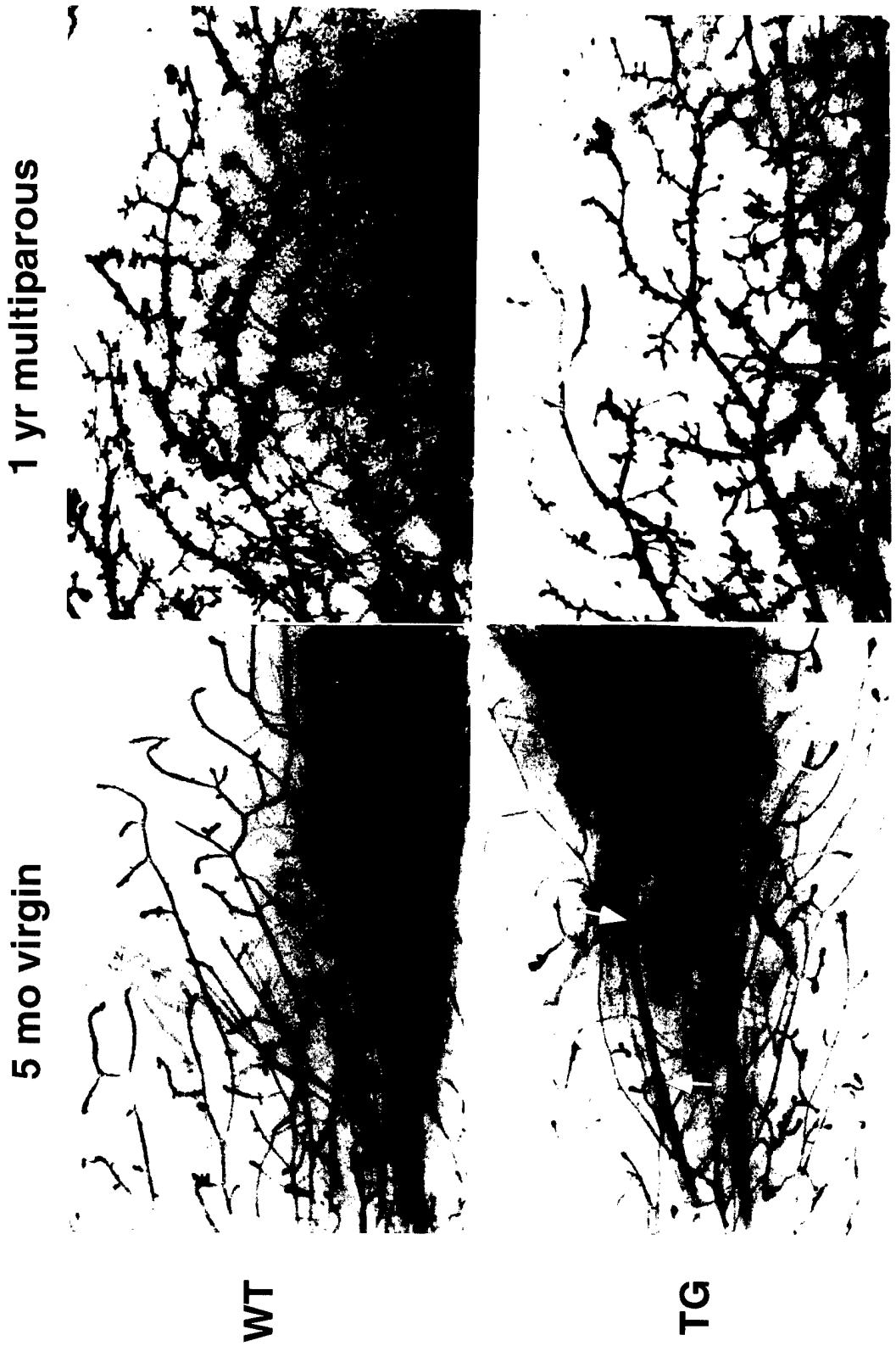

Figure 4

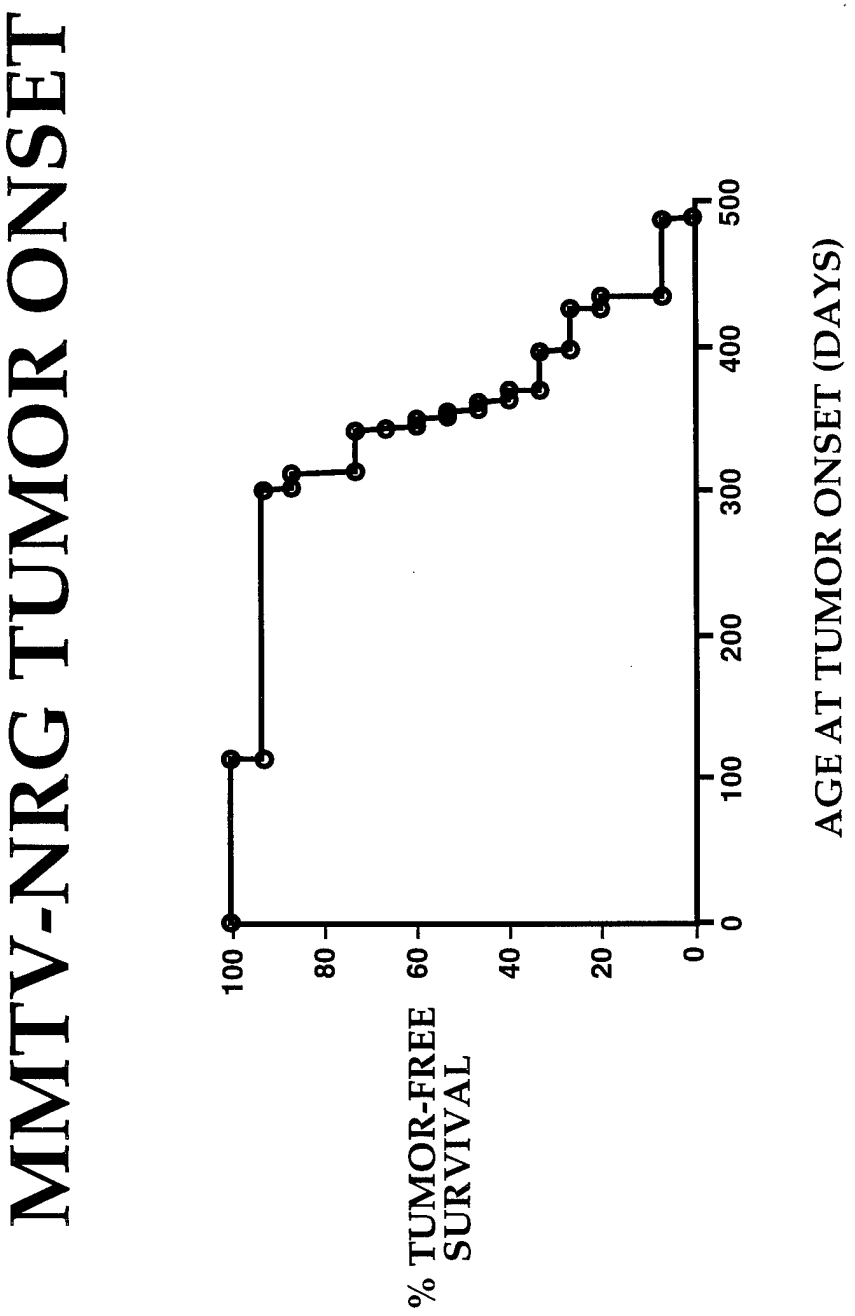
MMTV-NRG TUMOR ONSET
% TUMOR-FREE SURVIVAL
100
80
60
40
20
0
0
100
200
300
400
500
AGE AT TUMOR ONSET (DAYS)

Figure 5

NEUREGULIN TRANSGENE EXPRESSION IN MAMMARY GLAND TUMORS

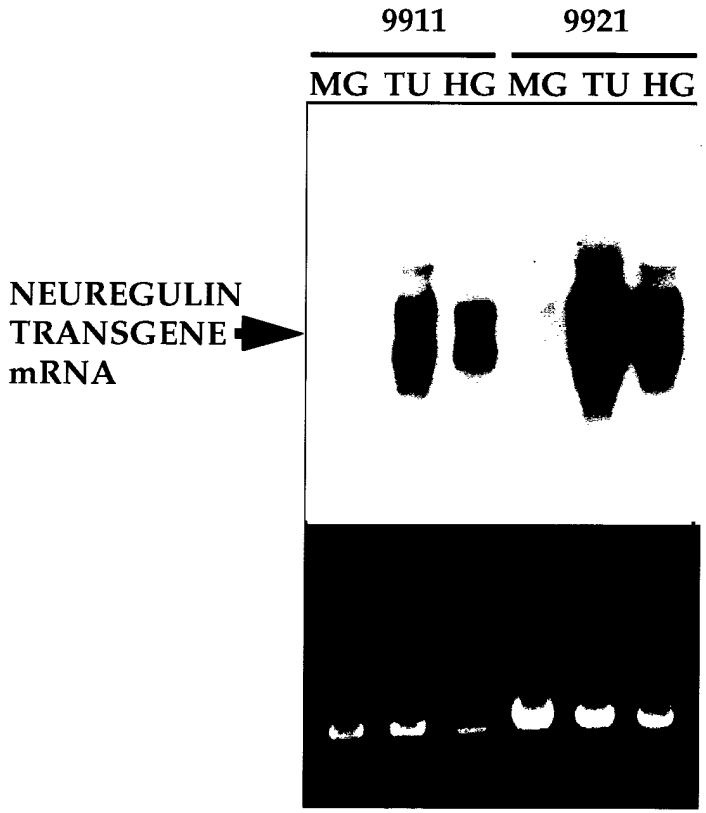

Figure 6

ERBB RECEPTOR EXPRESSION IN TUMOR DERIVED CELL LINES

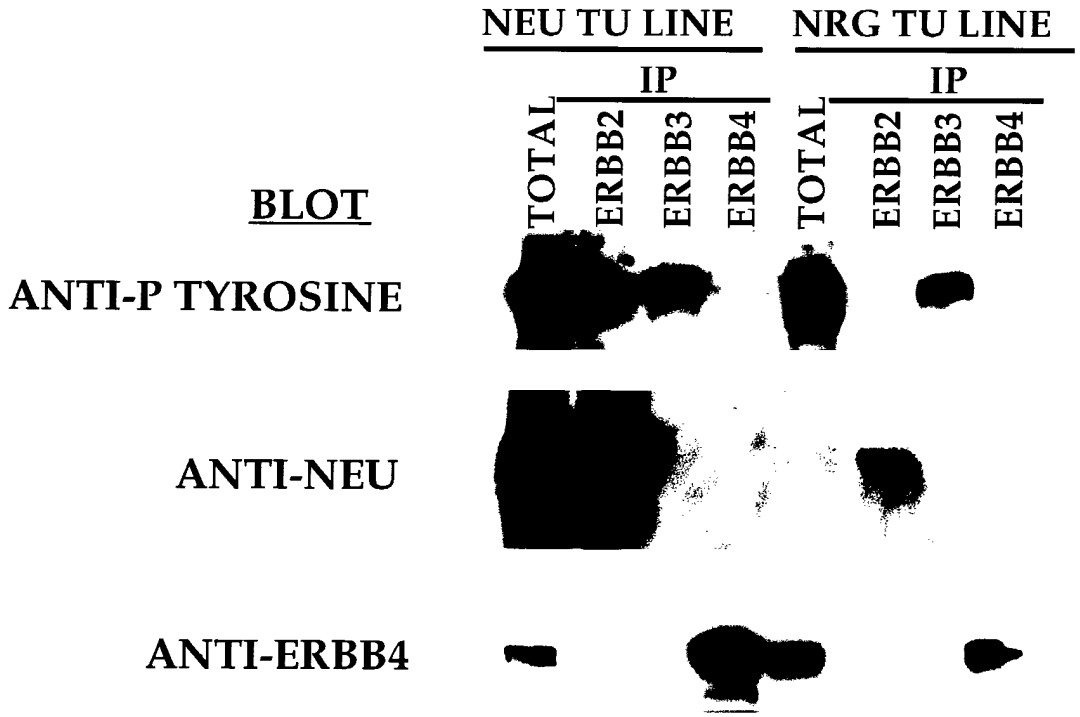

Figure 7

"a" ISOFORM EXPRESSION IN A NRG-INDUCED TUMOR CELL LINE

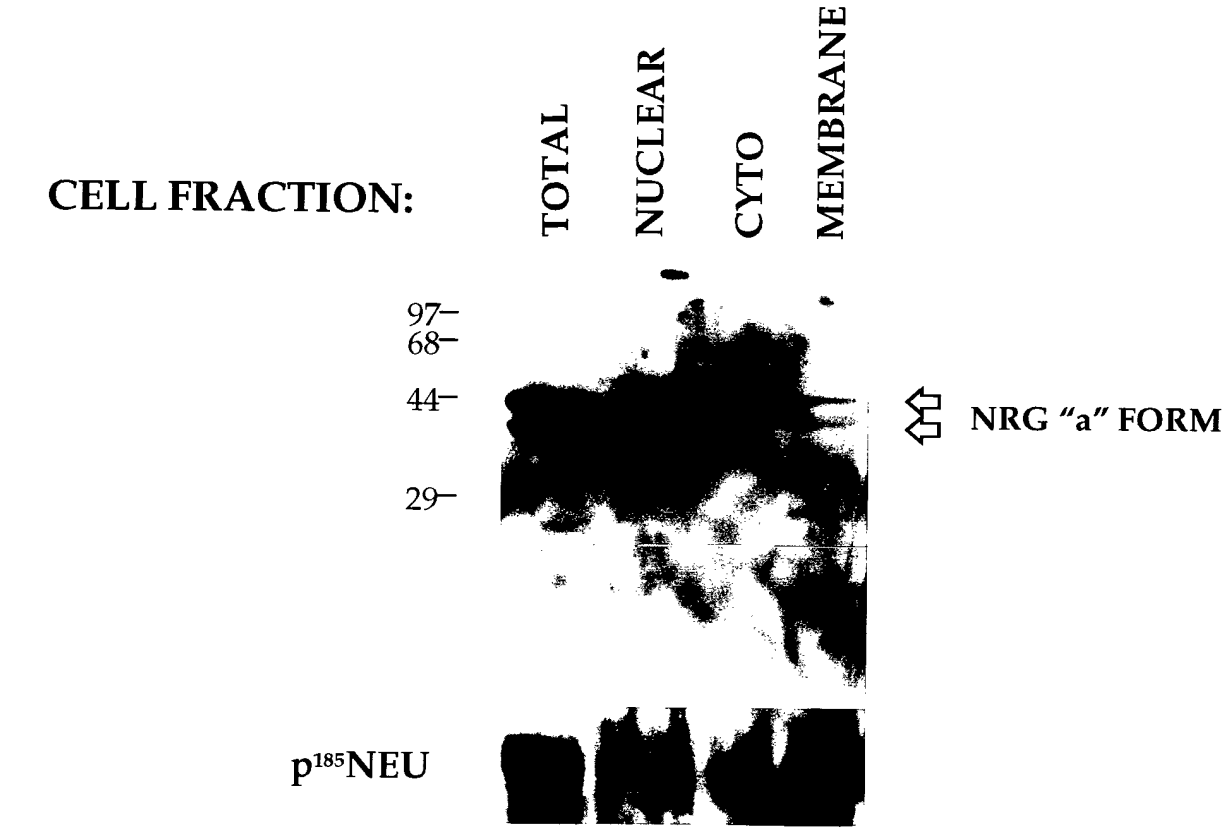

Figure 8

Figure 9